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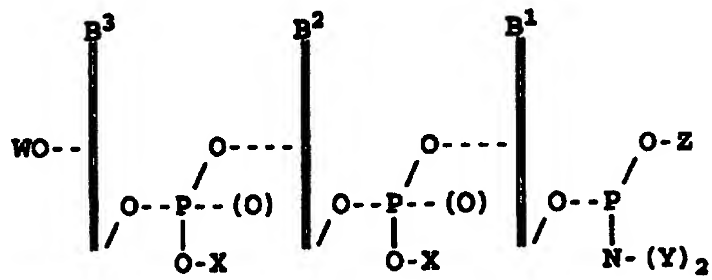
(57) Abstract

Compounds of formula (1) or (2) wherein W, X, Y and Z are each independently hydrogen or a protecting group; B^1, B^2 and B^3 are each independently a base selected from the group consisting of adenine, guanine, cytosine, thymine and uracil; and || is the residue of a ribose or deoxyribose, and methods for their synthesis and use are disclosed.



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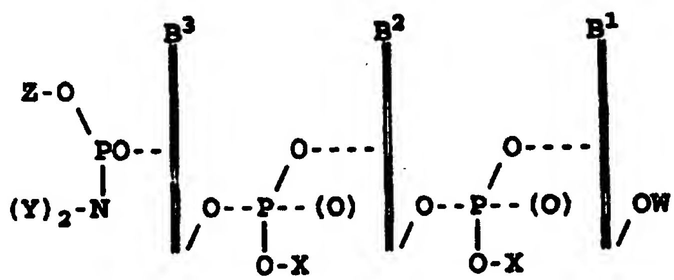

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CODON AMIDITES AND METHOD OF USING THEM TO PRODUCE OLIGONUCLEOTIDES AND MUTAGENESIS LIBRARIES

Technical Field

The invention relates to the synthesis of various types of oligonucleotides that are useful in analysis, therapy and other applications. More specifically, the invention concerns a technique for using preassembled 3'-phosphoramidite trinucleotides as building blocks to make oligonucleotides encoding a desired sequence of amino acids, optionally containing positions with random amino acids. Randomized DNA fragments, in particular, are useful in producing combinatorial libraries of peptides or proteins with a variety of binding properties, from which molecules of special interest can be selected.

In one popular format, a DNA fragment is synthesized corresponding to the sequence of a bacteriophage coat protein with an appended "scrambled region" where all possible codons will be presented. This fragment family is then inserted into phage DNA such that, in an appropriate host cell population, a library of different expressed peptide regions is created. Selection of phage with desirable characteristics can then be accomplished by a variety of criteria, and the sequence responsible can be determined by sequencing the appropriate portion of the phage DNA.

Background Art

Use of molecular biology techniques to encode randomized sets of short peptides, of interest for their own sake or as a means to modify specific sites within protein domains, are useful to select molecules with desirable binding specificities. Trinucleotides carrying certain protecting groups have long been used as intermediates to synthesize oligonucleotides corresponding to specific peptides of interest. For example, see Wakabayashi *et al.*, "Rapid Synthesis of Oligodeoxynucleotid s by using N-Methylimidazol as a Condensation Catalyst. Syntheses of Dodecanucleotides Corr sponding to Complementary Deoxyribonucleic Acid of the

Tetrapeptide Fragments of Cholecystokinin-Pancreozymin and Vasoactive Intestinal Peptide", Chem. Pharm. Bull., 30, 3951-58 (1982).

Certain dodecanucleotides have been synthesized on a solid polymer support with phosphate triester methods by elongation in the 3'-direction. The elongation can occur by condensing the 3'-phosphodiester with 5'-deblocked dinucleotides or trinucleotides having the 3'-(o-chlorophenyl)phosphoro-p-anisidate moiety by using 1-(mesitylene sulfonyl)-3-nitro-1H-1,2,4-tetrazole as the activating reagent. Ohtsuka *et al.*, "Deoxyribonucleic Acids and Related Compounds. VIII. Solid-Phase Synthesis of Deoxyribooligonucleotides with 3'-Modification by Elongation in the 3'-Direction", Chem. Pharm. Bull., 32, 85-93 (1984).

When adapted to solid phase synthesis, the phosphate triester method produces acceptable results for short fragments (10-20 bases in length), but not for the longer fragments needed to incorporate a scrambled sequence into a form suitable for insertion into a vector. Triester methods have largely been replaced by the phosphoramidite technique, which provides better performance in the synthesis of long fragments. For example, methods to increase the yield of long sequences prepared by solid phase synthesis have been reported using 3'-phosphoramidite functionalized, protected nucleotide dimers. Kumar *et al.*, "Improvements in Oligodeoxyribonucleotide Synthesis: Methyl N,N-Dialkylphosphoramidite Dimer Units for Solid Support Phosphite Methodology", J. Org. Chem., 49:25, 4905-4911 (1984).

Due to degeneracy in the DNA code, however, total scrambling of all four nucleotides (adenine, guanine, cytosine and thymine) at each site in a DNA sequence leads to more DNA species than peptide species, with the excess growing geometrically as a function of the length of the scrambled sequence. For a hexapeptide, the 64 million possible combinations of 20 amino acids expands to about 68 billion possible combinations of encoding oligonucleotides. Further, the inadvertent inclusion of stop codons sometimes results in

the formation of truncated peptide s qu nces, which furth r complicates the analysis.

Two major approaches have been described to overcome this redundancy problem. In a first approach, a semi-scrambled set of nucleotides is used to take advantage of the third-position redundancy in many codon assignments, for example, N-N-(G/C), which symbolizes a trinucleotide having in the first and second positions a random mix of all four nucleosides and, in the third position, a mix of only guanine and cytosine. This reduces the number of triplets from 64 to 32, while still encoding all 20 amino acids, and has only one stop codon.

However, each of the four nucleotides has a different rate of incorporation onto the tip of the growing oligonucleotide chain, so that the yield of the resulting triplets can vary as much as five-fold. Accordingly, to obtain a library containing all hexapeptides, the number of clones that must be examined is more than 15,000 times larger than the number indicated by simple combinatorial arithmetic.

In the second approach, individual codons are sequentially added to an oligonucleotide on a solid support, usually resin beads, which have been divided into separate containers. The contents of the containers are combined before proceeding to the next codon, for which the resin beads holding the growing chains are again separated into individual containers. This resin splitting approach does, in principle, produce a more nearly ideal final library, but requires a great deal of synthetic effort. Huse, U.S. Patent No. 5,274,563 issued 23 November 1993; and Glaser et *al*., "Antibody Engineering by Codon-based Mutagenesis in a Filamentous Phage Vector System", J. of Immun., 149:12, 3903-13 (1992). Specifically, handling problems, such as static electricity, make quantitative physical transfer of the beads difficult, and chemical inhomogeneity in the microsco ically settled beads can lead to poor coupling on some of the beads during solid phase synthesis.

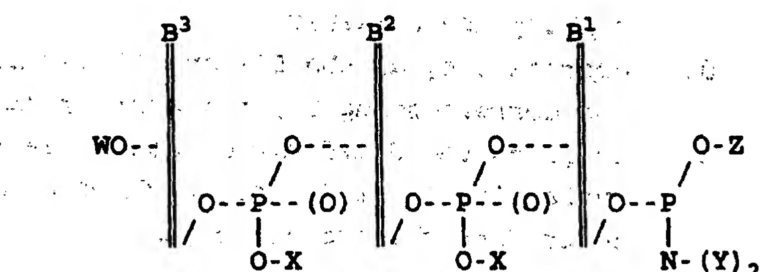
Furth r, to obtain reasonably uniform repr sentation of each peptide in a hexapeptide library with this approach, more than 64 million beads must b used to assur that the

random pathway taken by each bead through th different containers does not leave out some sequence by mathematical chance. A 1 μM synthesis on controlled pore glass (CPG) beads is typical for synthesis of DNA used in most molecular biology purposes. At this synthesis scale, however, only about 25,000 beads are present, which is too few to represent the entire library. Moreover, even if scale-up is attempted, it is a difficult procedure due to increasingly prohibitive handling problems and the attendant costs.

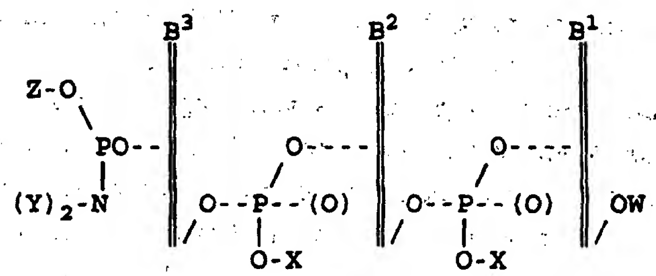
The Huse patent also speculates in passing that oligonucleotides could be synthesized from nucleotide triplets, but quickly discourages the use of this approach, stating that "the cost of synthesis from such triplets far exceeds that of synthesis from individual monomers because triplets are not commercially available." (See, Huse, column 2, lines 18-29) Unexpectedly, the materials and methods of the invention provide a less labor-intensive procedure than that used in the art and, at the same time, provides increased confidence that all the desired sequences will be available, even on a modest synthetic scale.

Disclosure of the Invention

The invention relates to compounds of the formula:



or



wherein W, Y and Z are each independently hydrogen r a protecting group;

X is $-CH_2CH_2CN$;

B^1, B^2 and B^3 are each independently a base selected from the group consisting of protected adenine, protected guanine, protected cytosine, protected or unprotected thymine and protected or unprotected uracil; and

‖ is the residue of a ribose or deoxyribose.

Further, a method has been found for making these compounds and, further, compounds wherein X may be hydrogen or any suitable protecting group, comprising the steps of:

a. treating a nucleoside comprising a base, a ribose or deoxyribose residue, and a first protecting group at the 5′ position or the 3′ position, with a trialkylsilyl halide to produce the corresponding 3′- and 5′-substituted nucleoside;

b. removing the first protecting group at the 5′ or 3′ position to produce a 5′ or 3′ deprotected, 3′ or 5′-substituted nucleoside;

c. coupling the 5′ or 3′ deprotected 3′ or 5′ substituted nucleoside with a first nucleoside 3′ or 5′ phosphoramidite and then oxidizing to form a phosphate triester;

d. deprotecting at the 5′- and 3′-termini to give the corresponding 3′,5′-dihydroxy dinucleoside;

e. coupling the dihydroxy dinucleoside with a second nucleoside 3′ or 5′ phosphoramidite and oxidizing to produce the two corresponding 3′ or 5′ hydroxy trinucleotides;

f. separating away unwanted products; and

g. converting the 3′- or 5′-hydroxy trinucleotide to a 3′- or 5′ phosphoramidite.

In another embodiment, a panel is made up of from two to twenty of these 3′- or 5′-phosphoramidite trinucleotide codons or their complements, wh re each codon encodes a different amino acid.

Three processes for synthesizing an oligonucl otid are disclosed. To synthesiz an oligonucleotide encoding a

sequence of "n" pre-determined amino acids, or its complement, the process of the invention comprises the steps of:

(a) coupling a trinucleotide codon, or its complement, onto a solid support;

(b) sequentially condensing, through a phosphoramidite linkage, with an immediately preceding codon "n-1" trinucleotide codons or their complements, each codon or its complement corresponding to the next pre-determined amino acid in the sequence; and

(c) cleaving the oligonucleotide from the solid support.

The automated solid phase supported chemistry used in the invention can include a large number of materials generally known to those of ordinary skill in the art. Examples of such useful materials includes nucleoside-functionalized CPG, i.e., functionalized with deoxyribose cytosine (dC) or another nucleoside, e.g., dG, dU or rC; tetrazole activator solution; acetylating capping solutions; iodine oxidation solutions; dichloroacetic acid deprotecting solvent; and automated DNA synthesizers.

To synthesize an oligonucleotide encoding a peptide having at least one pre-determined amino acid position and at least one random amino acid position, a second synthetic process of the invention comprises the steps of:

(a) coupling a first trinucleotide codon, or the complement to said codon, onto a nucleoside- or nucleotide-bearing solid support;

(b) for each pre-determined amino acid position, sequentially coupling through a phosphoramidite to an immediately preceding codon, or complement, a trinucleotide codon, or its complement, corresponding to the pre-determined amino acid;

(c) for each random amino acid position, coupling to an immediately preceding codon or complement, a mixture of from two to twenty trinucleotide codons, at least two of the trinucleotides

representing a codon to a diff rent amino acid; and

(d) cleaving said nucleotide from the solid support.

Steps (b) and (c) of this process are combined in such a way that each pre-determined codon sequentially corresponds to each pre-determined amino acid position in the oligonucleotide.

Further, the identity and ratio of codons used in each such mixture can be made to represent the desired degree of diversity in the corresponding random amino acid position. Thus, if reduced coupling efficiency occurs with particular codons making up the desired oligonucleotide, this defect can be readily overcome by minor adjustments in the relative concentrations of individual codons. For example, dC-functionalized CPG or rC supports may be prone to give unequal incorporation of bases when mixtures are used, perhaps due to steric hindrance. With the invention, this inequality can be compensated for by varying the relative concentrations of the various codons.

To synthesize an oligonucleotide having at least one region of random amino acid positions, the third synthetic process of the invention comprises the steps of:

(a) coupling at least one pre-existing trinucleotide codon or complement onto a nucleoside- or nucleotide-bearing solid support;

(b) for each random amino acid position, sequentially coupling through a phosphoramidite to the immediately preceding codon, or complement, a mixture of from two to twenty pre-existing trinucleotide codons, each codon corresponding to a different amino acid; and

(c) cleaving the oligonucleotide from the solid support.

In this process, the identity and ratic of codons used in each such mixture are representative of the degree of diversity desired in the corresponding random amino acid position.

Brief Description of the Drawings

The present invention will be more clearly understood by referring to the following drawings, in which

Figure 1A shows a SAX HPLC trace of 16-mer oligonucleotide dATACTTARCATGAGCC made with the codon amidites of the invention;

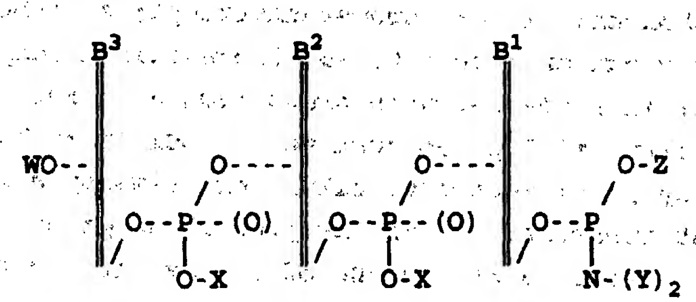
Figure 1B shows a SAX HPLC trace of the same 16-mer oligonucleotide dATACTTARCATGAGCC made with amidite monomers; and

Figure 1C shows a SAX HPLC trace of the result when both the fragment of Figure 1A and the fragment of Figure 1B are co-injected.

Figure 2 shows the results of analysis by reverse phase HPLC of coupling a codon amidite mixture to dC support.

Modes of Carrying Out the Invention

The compounds of the invention have the formula:



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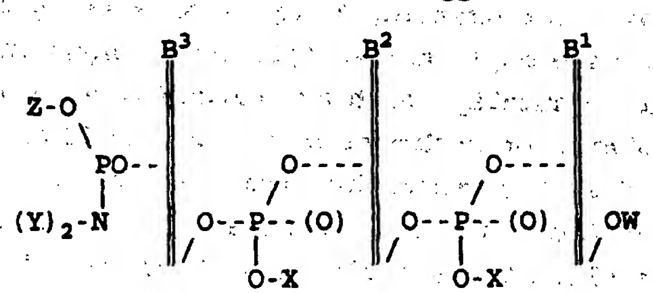


as shown and described above.

W, X, Y and Z in the above formula are each independently hydrogen or a protecting group. Preferably, the protecting group is selected such that the reaction between the prot cting group moiety and the atom or atoms at the position

being protected is a high-yield step. Further, all of the protecting groups used should be stabl to the usual reacti n conditions, such as are used in condensation reactions, to further manipulate the protected compound.

While a protecting group at the 3'-position may desirably be capable of selective cleavage without detrimental effect on a protecting group in the 5'-position, it should be noted that this is not always a necessary feature of the process of the invention. Specifically, in the process of the invention, both the 3'- and the 5'-positions of the dimer intermediates are deprotected, usually simultaneously. Subsequent reaction forms mixtures from which unwanted components are removed.

W can be hydrogen or any one of a wide variety of protecting groups so long as they can be removed independently of the other protective groups (X, Y and Z). Preferably, when W is a protecting group, it is a triphenylmethyl group, such as DMT (dimethoxytrityl) or monomethoxytrityl; a carbonyl-containing group such as FMOC (9-fluorenylmethyloxycarbonyl) or levulinoyl; an acid-cleavable group such as pixyl; a fluoride-cleavable alkylsilyl group such as t-BDMSi (tert-butyl dimethylsilyl), triisopropyl silyl, or trimethylsilyl; and the like. Most preferably, W is the protecting group DMT.

X may be H or any one of a wide variety of protecting groups, so long as it can be removed without destroying the product nucleotide. For example, X may be an alkyl group, such as methyl, ethyl, isopropyl, tert-butyl, or n-hexyl; haloalkyl such as haloethyl; cyanoalkyl such as $-CH_2CH_2CN$; an aryl group such as o-chlorophenyl or methoxyphenyl; and the like. However, most preferably, X is the cyanoalkyl group $-CH_2CH_2CN$.

Y may be H or any one of a wide variety of groups. However, as a guideline to selecting useful groups, Y should preferably be a hydrocarbon. For example useful Y groups include alkyl groups such as methyl, ethyl, isopropyl, tert-butyl, or n-hexyl. Alternatively, tw Y groups, taken together, may form a heter cyclic ring with the nitrogen atom protected, such as morpholino, piperidino, pyrollidino, and th like. Most preferably, Y is an alkyl group, such as methyl or isopropyl.

Z can be any on of larg number of diff r nt protecting groups and should be chosen so as to have the same characteristics as X. Suitable examples of Z as a protecting group include an alkyl group, such as methyl, ethyl, isopropyl, tert-butyl, or n-hexyl; a cyanoalkyl group such as $-CH_2CH_2CN$; an aryl group such as o-chlorophenyl, or methoxyphenyl; and the like. Preferably, Z is cyanoalkyl such as $-CH_2CH_2CN$.

In a particularly preferred embodiment, W is DMT, X and Z are each $-CH_2CH_2CN$, and Y is an isopropyl group. Methods of manipulating various protective groups with respect to DNA are known to those of ordinary skill in the art. Using similar methods to protect the 2'-hydroxy group of an RNA molecule with a protecting group is also known, e.g., see Wang et al., "Enzymatic and NMR Analysis of Oligoribonucleotides Synthesized with 2'-tert-Butyldimethylsilyl Protected Cyanoethylphosphoramidite Monomers", Nucleic Acids Research, 18:11, 3347-52 (1990).

The bases B^1, B^2 and B^3 are each independently selected from the group consisting of protected adenine, protected guanine, protected cytosine, protected or unprotected thymine and protected or unprotected uracil. When any one or more of B^1, B^2 and B^3 is adenine, cytosine or guanine, it should be protected with a group such as benzoyl, isobutyryl, phenoxyacetyl, methoxyacetyl, an amidine, or the like.

In a particularly preferred embodiment, B^1 is selected from the group consisting of an adenine base protected with a benzoyl protecting group, a thymine base, a cytosine base protected with a benzoyl protecting group, and a guanine base protected with an isobutyryl protecting group. In another preferred embodiment, B^2 is selected from the group consisting of thymine and guanine and, when B^2 is guanine, it is protected with an isobutyryl protecting group.

In yet another preferred embodiment, B^3 is selected from the group consisting of protected adenine and cytosine. When B^3 is either adenin or cytosine, it is preferably protected with a b nzoyl protecting group.

Pr ferred groupings of B^1, B^2 and B^3, with protective groups often preferred for each grouping, are shown below:

$B^3 = A^{bz}$; $B^2 = T$; and $B^1 = A^{bz}$

$B^3 = T$; $B^2 = T$; and $B^1 = C^{bz}$

$B^3 = C^{bz}$; $B^2 = T$; and $B^1 = A^{bz}$

$B^3 = G^{ibu}$; $B^2 = T$; and $B^1 = A^{bz}$

$B^3 = C^{bz}$; $B^2 = G^{ibu}$; and $B^1 = A^{bz}$

"‖" in the above formula represents the residue of a ribose or deoxyribose. Ribose is the pentose $CH_2OH(CHOH)_3CHO$ that forms the sugar backbone of an RNA polynucleotide chain in its furanose form by a series of 5'-3' sugar-phosphate links. Deoxyribose has one less hydroxy group at the 2-position of the sugar ring and is the sugar that forms the backbone of a DNA chain.

The compounds of the invention can be made by:

a. treating a nucleoside comprising a base, a ribose or deoxyribose residue, and a first protecting group at the 5' position or the 3' position, with a trialkylsilyl halide to produce the corresponding 3'- and 5'-substituted nucleoside;

b. removing the first protecting group at the 5' or 3' position to produce a 5' or 3' deprotected, 3' or 5'-substituted nucleoside;

c. coupling the 5' or 3' deprotected 3' or 5' substituted nucleoside with a first nucleoside 3' or 5' phosphoramidite and then oxidizing to form a phosphate triester;

d. deprotecting at the 5'- and 3'-termini to give the corresponding 3',5'-dihydroxy dinucleoside;

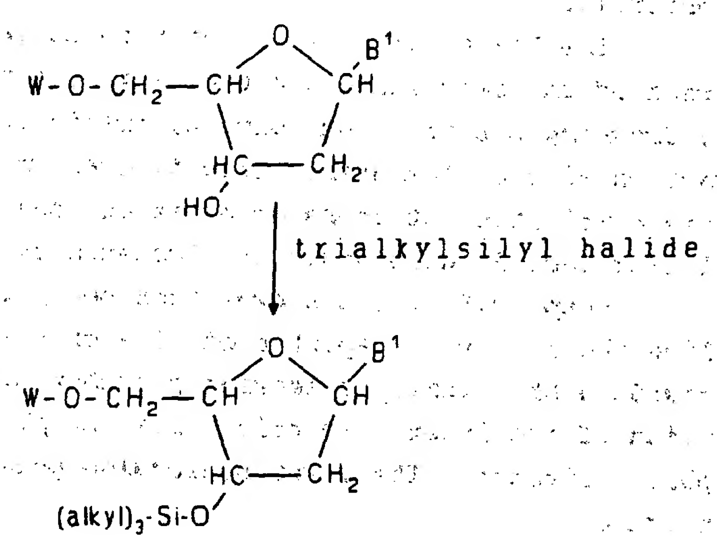
e. coupling the dihydroxy dinucleoside with a second nucleoside 3' or 5' phosphoramidite and oxidizing to produce the two corresponding 3' or 5' hydroxy trinucleotides;

f. separating away unwanted products; and

g. converting the 3'- or 5'-hydroxy trinucleotide to a 3'- or 5' phosphoramidite.

Step "a." f this particularly advantageous process for making the compounds of the inv ntion comprises treating the starting nucleoside, which has a 3'- or 5'-protecting group such

as DMT, with a trialkylsilyl halide, such as t-butyl dimethylsilane halide. For example, when the starting nucleoside has a 5'-protecting group, the product will be a 3'-trialkylsilyl, 5'-protected nucleoside. This reaction is illustrated below:



where W and B_1 are as defined above.

This reaction is typically carried out in the presence of $AgNO_3$ or imidazole, which acts as a catalyst, and an organic solvent, such as pyridine or tetrahydrofuran. Preferably, the reaction takes place in an inert atmosphere, such as that provided by nitrogen or argon gas. The product may be isolated from the rest of the reaction mixture by any convenient method, such as by drowning out in a non-solvent, precipitating out, extraction with an immiscible liquid, evaporation of a solvent, or some combination of these or other methods. A particularly preferred method of adding a 3'-t-butyl dimethylsilyl protecting group is provided by Ogilvie *et al.*, Pure and Appl. Chem., 59, 325-30 (1987).

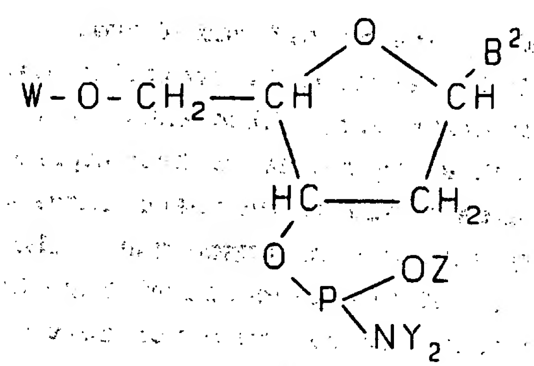
In step "b.", the protecting group at the 5'- or 3'-position can be removed selectively by any one of a number of various procedures, for example, by treatment with a mineral acid, such as HCl or H_2SO_4; an organic acid such as HOAc, dichloroacetic acid, trichloroacetic acid, b nzenesulfonic acid; anoth r strong acid; a metal halid such as $ZnBr_2$ or another

Lewis acid; a bas such as piperadine or hydrazine; and the like.

In the removing step "b.", a solvent is typically used, such as an alcohol, acetonitrile, diethyl ether, acetone, ethyl acetate, or the like. Preferably, the solvent is acetonitrile.

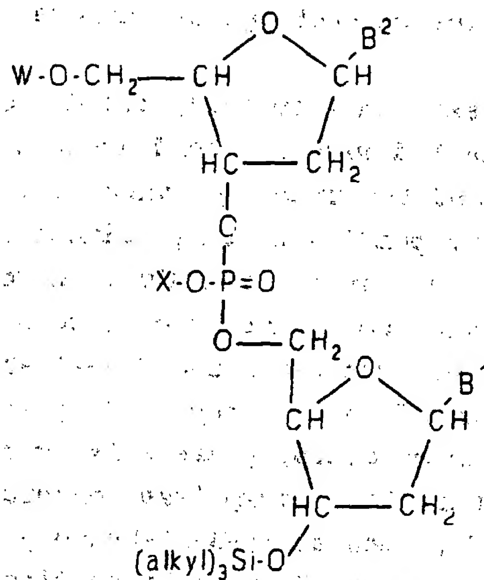
The 3'- or 5'-deprotected product may be isolated from the rest of the reaction mixture by any convenient method, such as by drowning out in a non-solvent, precipitating out, extraction with an immiscible liquid, evaporation of a solvent, or some combination of these or other methods. Preferably, the 3-silyl-protected nucleosides are isolated by crystallization.

Step "c." of the above-described process involves coupling the 5'- or 3'-deprotected, 3'- or 5'-substituted nucleoside with a first nucleoside phosphoramidite, followed by oxidation of the internucleotide trivalent phosphorous to form a phosphate triester. The first nucleoside phosphoramidite has the formula:



where W, Y, Z and B^2 are as defined above. Such phosphoramidites are usually available commercially under the trade name "DNA Amidites." Typically, the nucleoside phosphoramidite is used in the amount of from about 1.0 to about 1.1 equivalents, most preferably about 1.1 equivalents.

After being condensed with, for example, the 5'-deprotect d and 3'-substitut d nucleoside described above, and after the dinucleotide ph sphite triesters have been oxidized to phosphate triesters, th coupled product has the formula:



where W, X, B^1 and B^2 are as defined above.

This dimer formation reaction typically takes place in the presence of a tetrazole for the purpose of activating the phosphoramidite. The amount of tetrazole present can vary widely between about 5 to about 10 equivalents, but preferably is about 10 equivalents. Other materials that can be substituted for tetrazole include benzotriazole and p-nitrophenyl tetrazole.

The dinucleotides are then given a deblocking procedure, such as treatment with acid, to deprotect the 5'- and 3'-termini, using one of the deblocking procedures described above, either alone or in combination with each other or other procedures known to those of ordinary skill in the art. The resulting deprotected 3',5'-dihydroxy dinucleoside is typically isolated from the rest of the reaction mixture by any convenient method, such as by drowning out in a non-solvent, precipitating out, extraction with an immiscible liquid, evaporation of a solvent, or some combination of these or other methods. A preferred isolation method is by chromatography

Further, when a silyl protecting group is present in step "d.", a silyl cleaving agent such as KF is also typically added to the reaction mixture, primarily to diminish side reactions but also to accelerate the deprotecting reaction.

Other examples of such agents include tetrabutylammonium fluoride.

Next, in step "e.", the deprotected 3',5'-dihydroxy dimer is coupled with a second nucleoside phosphoramidite having a base B^3, and the trimer product is then oxidized to produce the two corresponding hydroxy trinucleotides. In a preferred embodiment, the deprotected dimer is coupled with the second phosphoramidite to produce the corresponding 5'-W-3'-hydroxy trinucleotide. The resulting 5'-W-3'-hydroxy trinucleotide can then be isolated, as in step "e." by any means for separating away unwanted products, preferably by column chromatography. The final step, "g.", comprises converting the 3'- or 5'-hydroxy trinucleotide, such as 5'-W-3'-hydroxy trinucleotide, to the corresponding 3'- or 5-'phosphoramidite, preferably by the procedure of Sinha *et al.*, Tett. Lett., 24, 5843-46 (1983), and isolating this by column chromatography.

The resulting 3'- or 5'-phosphoramidite trinucleotides, also called codon amidites, can be coupled onto nucleoside or nucleotide-bearing solid supports with semi-manual methods. For example, a Biosearch 8750 four-column, automated DNA synthesizer running a synthesis program without the coupling step can be used for 5'-deblocking, oxidation and capping, as well as washing between steps.

Further, a mixture of from two to twenty 3'- or 5'-phosphoramidite trinucleotide codons, or their complements, can be used to assemble a panel of codons, with each codon encoding a different amino acid. Such panels are useful for making oligomers, such as synthetic DNA fragments, by sequentially coupling the individual codons in the panel to a dC nucleoside- or nucleotide-functionalized support.

Specifically, in accordance with one of the processes of the invention, an oligonucleotide can be synthesized to encode a sequence of "n" pre-determin^d amino acids, or its complement. Such a process comprises the steps of:

(a) coupling a trinucleotide codon or its complement nto a s lid support;

(b) sequentially condensing, through a phosphoramidite linkage, with each immediately

preceding cod n, or complement, n-1 trinucleotide codons or their complements, each codon or its complement corresponding to the next pre-determined amino acid in the sequence; and

(c) cleaving the oligonucleotide from the solid support.

Step (b) above, directed to sequential condensation, is as described above generally for condensation reactions. Step (a), the coupling of a codon onto a solid support, and step (c), the cleavage of the resulting oligonucleotide from the support, are performed by methods known to those of ordinary skill in the art. The cleavage procedure usually removes all protecting groups simultaneously to generate biologically active material.

An oligonucleotide encoding a peptide having at least one pre-determined amino acid position and at least one random amino acid position can be prepared in accordance with the invention by a process comprising the steps of:

(a) coupling a first trinucleotide codon, or the complement to said codon, onto a nucleoside- or nucleotide-bearing solid support;

(b) for each pre-determined amino acid position, sequentially coupling through a phosphoramidite to an immediately preceding codon, or complement, a trinucleotide codon, or its complement, corresponding to the pre-determined amino acid;

(c) for each random amino acid position, coupling through a phosphoramidite to an immediately preceding codon, or complement, at least two of the trinucleotides representing a codon, at least two of the trinucleotides representing a codon to a different amino acid; and

(d) cleaving said nucleotide from the solid support.

Each pre-determined codon sequentially corresponds to each pre-determined amino acid position in the desired oligonucleotide. Furth r, steps (b) and (c) above ar preferably combined in such a way that the identity and ratio of the codons used in each said mixture represent the degree of diversity desired in the corresponding random amino acid position.

Finally, in accordance with a third embodiment of the invention, an oligonucleotide having at l ast one region f random amino acid positions, which may be optionally preceded or followed by "n" pre-determined flanking amino acid sequences, or their complements, the third synthetic process of the invention comprises the steps of:

(a) coupling at least one pre-existing trinucleotide codon, or complement, onto a nucleoside- or nucleotide-bearing solid support;

(b) for each random amino acid position, sequentially coupling through a phosphoramidite to the immediately preceding codon, or complement, a mixture of from two to twenty pre-existing 3'-phosphoramidite trinucleotide codons, each codon corresponding to a different amino acid; and

(c) cleaving the oligonucleotide from the solid support.

The identity and ratio of codons used in each mixture represent the degree of diversity desired in the corresponding random amino acid position.

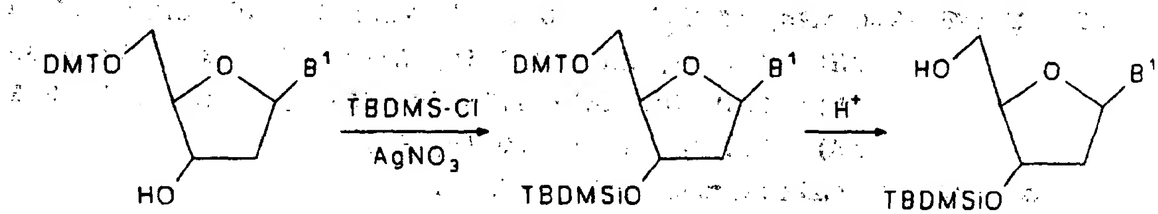
These processes of the invention for making oligonucleotides provide new methods for building diverse DNA libraries, using the pre-formed triplet codons in combination with reliable phosphoramidite DNA synthesis. Although coupling efficiencies may be modest, the amplification power of the polymerase chain reaction (PCR) renders them adequate for a wide range of purposes. For example, combinatorial libraries using a systematically diversified sets of monomers have been made by chemical means. Using the codon amidites of the invention, the advantages of a practical synthetic pathway and the ability to thoroughly characterize the resulting compounds can now be extended to recombinant libraries. Selected subsets of amino acids can be used to analyze structure/function relationships in a wide variety of contexts, such as the role of steric bulk versus electronic properties in a particular chemical environment. Specifically, the striking biases that exist in amino acids present at short distances from bound ligands have

already be n noted in a collection of about 50 protein crystal structures, thus enabling the synthesis of novel binding sites by directed mutagenesis. This, in turn, results in the ability to produce more selectively targeted biological materials such as drugs and diagnostic markers.

It is therefore seen that the compositions and processes of the invention are superior to the generation of codons using mixtures of monomers to obtain a random mix of amino acids in a particular position, because (1) stop codons are eliminated; (2) the codons expressed are more easily controlled, particularly when the expression of a library containing less than 20 amino acids at a given site is desired; and (3) the codon redundancy inherent in the standard N-N-(G/C) mixed site is eliminated since an inflated library size is not needed to achieve complete representation. Moreover, the compositions and processes of the invention are clearly superior to the physical support-splitting methods, which involve repetitive coupling, mixing, and dividing steps, since less labor is required. Further, only a modest synthesis scale is needed to provide all of the desired sequences.

The following examples are intended to illustrate but not to limit the invention.

Example 1: **Preparation of N^4-Benzoyl-3'-tert-butyl-dimethylsilyl Deoxycytidine**



where B^1 is C^{bz}.

A solution of 5 g (7.9 mmol) N^4-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine in 200 mL of pyridine was reduced to a foam *in vacuo*. Another 200 mL of pyridine and 2.5 g of $AgNO_3$ was added, and about 50 mL of the pyridine solvent was removed by rotary evaporation. The flask containing the reaction mixture was flushed with argon, and 3 g of the solid

tert-butyldimethylsilyl chloride (TBDMS-Cl) (19.8 mmol) was added. The resulting s luti n was allowed to stand overnight.

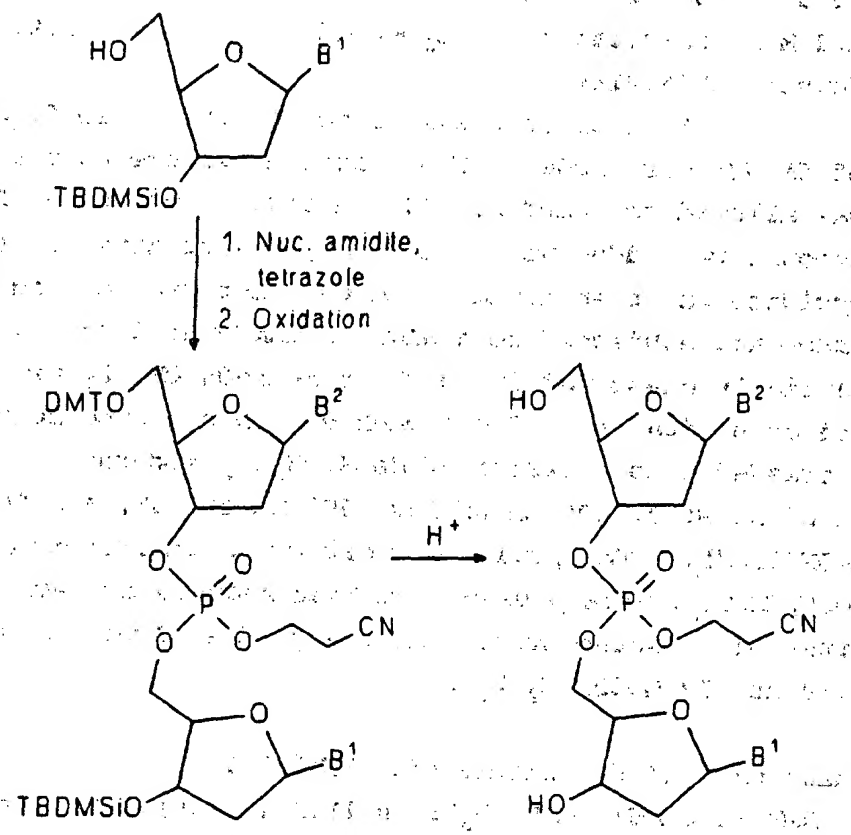
The next day, 10 mL of methanol was added, and the reaction mixture was reduced to a solid *in vacuo*. 200 mL of ethyl acetate and 100 mL of water were added, and the mixtur was transferred to a separatory funnel and allowed to separat . After separating, the organic layer was washed with 100 mL of water and dried over anhydrous Na_2SO_4. The solution was reduced to a foam and subjected to high vacuum overnight. The yield was 5.8 g (98%) of a single spot material (rf 0.81 in 9:1 CH_2Cl_2:MeOH).

This 3'-protected product was dissolved in 200 mL CH_3CN, and 10 mL of 6N HCl was added. The reaction mixture turned red, was allowed to stand for one minute, and then was neutralized to a colorless endpoint by adding 28% aqueous ammonia. The solution was reduced to a solid *in vacuo*. The solid was dissolved in 200 mL of methanol. When 100 mL of water were added, the solution became cloudy. The mixture was placed in a separatory funnel and washed with 200 mL of hexane. Crystals formed in the aqueous layer, and these were collected by filtration and washed with hexane. The aqueous layer was then chilled, and the resulting crystals were combined with those previously obtained to give 2.5 g of white powder (73% yield; rf 0.64; mp 105-110°C).

^{1}H NMR (300 mHz, $CDCl_3$): δ 8.3 (d, 1H); 7.8 (d, 2H); 7.5 (d, 2H): 7.4 (5, 3H); 7.2 (s, 1H); 6.15 (s, 1H); 4.45 (m, 1H); 3.95 (s, 2H); 3.75 (s, 2H); 2.4 (m, 1H); 2.3 (m, 1H); 0.8 (s, 9H); 0.0 (s, 6H).

m/z 469.7, calculated. MNa^+ 469.7.

Example 2: **Preparation of Thymidine-3'-yl N^4-Benzoyl-deoxycytidine-5'-yl Cyanoethylphosphate**



where B^1 is C^{bz} and B^2 is T.

A solution of 1 g (2.2 mmol) N^4-benzoyl-3'-tert-butyldimethylsilyl deoxycytidine in 150 mL of dry CH_3CN was reduced to a foam *in vacuo*. The procedure was repeated, and the residue was subjected to high vacuum for one hour. About 40 mL of dry CH_3CN was added and, after the solid starting material dissolved, 2 g (2.7 mmol) of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite was added. To this was added 10 mL of 0.5 N tetrazole in dry CH_3CN, and the mixture was allowed to stand for 15 minutes. 150 mL of ethyl acetate, 50 mL of water, 100 μL of pyridine, and 2 g of iodine were added, and the mixture was shaken until an orange color persisted. To this was added 4 g of $Na_2S_2O_3$, and the mixture was shaken in a separatory funn l until the orange color disappeared. The layers were s parated, and the organic phase was washed with 200 mL of a

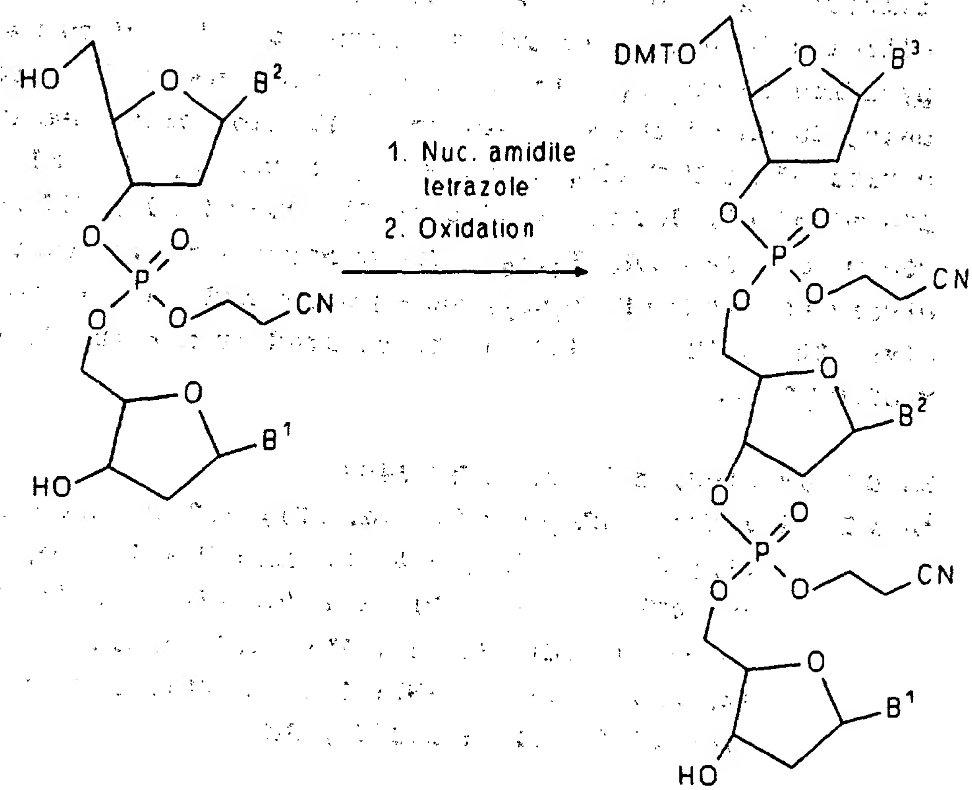
saturated solution of $NaHCO_3$ in water and dried over anhydrous Na_2SO_4. The solution was filtered and reduced to a foam *in vacuo*. The yield was 2.7 g (100%; rf 0.6 in 10% $MeOH/CH_2Cl_2$). H.R.M.S. calculated for $C_{56}H_{66}N_6O_{14}SiP$: 1105.4144. Found: 1105.4164.

This product was dissolved in 100 mL CH_3CN, and 10 mL of 6N HCl were added. The reaction mixture turned orange and was allowed to stand for 90 minutes. Cautiously, 28% aqueous ammonia was added until the orange color disappeared. 5 mL of pyridine was then added. The solution was reduced to a solid *in vacuo* and subjected to a high vacuum overnight. The solid was partially dissolved in 40 mL of 5% $MeOH/CH_2Cl_2$ and applied to a bed of silica (4 X 15 cm) packed with the same solvent. Elution proceeded with a series of $MeOH/CH_2Cl_2$ mixtures, as follows: 500 mL of 5% MeOH, 500 mL of 10%, 500 mL of 15%, and 500 mL of 20% $MeOH/CH_2Cl_2$. The product-containing fractions eluted at 20% $MeOH/CH_2Cl_2$, were pooled, and then reduced *in vacuo* to give, after high vacuum overnight, 1.2 g of a clear tar (68% yield; rf 0.19 at 10% $MeOH/CH_2Cl_2$).

MALDI m/z 711.6, calculated. MNa^+ 711.

^1H NMR (400 mHz, DMSO D_6): δ 11.2 (s, 1H); 11.1 (m, 1H); 8.1 (d, 1H); 7.9 (d, 2H); 7.6 (s, 1H); 7.5 (t, 1H); 7.4 (t, 2H); 7.2 (s, 1H); 6.1 (m, 2H); 5.4 (d, 1H); 5.1 (m, 1H); 4.9 (m, 1H); 4.1 (m, 5H); 3.9 (m, 2H); 3.5 (m, 2H); 2.8 (5, 2H); 2.3 (m, 3H); 2.0 (m, 1H).

Example 3: Preparation of N^6-Benzoyl-5'-O-(4,4'-dimethoxytrityl)d oxyadenosine-3'-yl Cyanoethylphosphate-5'-yl Thymidine-3'-yl N4-Benzoyldeoxycytidine-5'-yl Cyanoethylphosphate



where B^1 is C^{bz}, B^2 is T, and B^3 is A^{bz}.

A solution of 1.2 g (1.4 mmol) of thymidine-3'-yl N^4-benzoyldeoxycytidine-5'-yl cyanoethylphosphate in a mixture of 100 mL dry CH_3CN and 10 mL DMSO was reduced to a volume of about 10 mL *in vacuo*. This process was repeated with 100 mL of dry CH_3CN.

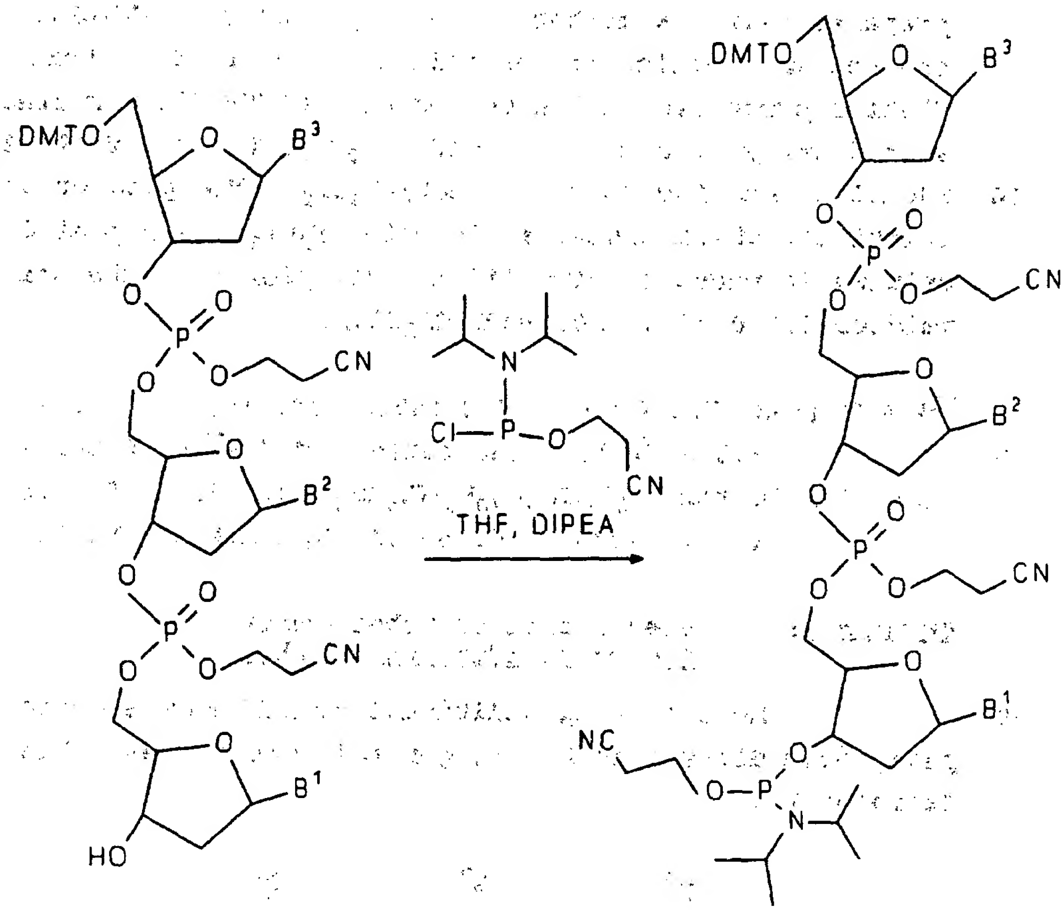
After flushing with argon, 60 mL dry CH_3CN and 10 ml of 0.5 N tetrazole in CH_3CN were added. Three aliquots of 500 mg N^6-benzoyl-5'-O-(4,4'-dimethoxytrityl)-deoxyadenosine-3'-O-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite were added at 10 minute intervals (total 1.5 g, 1.7 mmol). After 10 minutes, 150 mL of ethyl acetat and 50 mL of water were added, along with 100 μL of pyridine. About 1 g of iodine was then added, and the flask was shaken until an orange color persisted. To

this was added 4 g of $Na_2S_2O_3$, and the mixture was shaken until the orange color disappeared. The lay rs were separated, and the organic phase was washed with 200 mL of saturated $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. The mixture was filtered, and the filtrate was reduced to a foam *in vacuo*. A silica column (3 x 12 cm) was prepared using 2% methanol and 1% pyridine in CH_2Cl_2. The trimer product was applied to the column using 20 mL of the same solvent. Elution proceeded with a series of $MeOH/CH_2Cl_2$ mixtures, specifically, 200 ml of 2% MeOH, 200 ml of 4%, 200 mL 6%, 200 mL 8%, 200 mL 10%, 200 mL 12%, and 200 mL of 14% $MeOH/CH_2Cl_2$. The product-bearing fractions, which eluted at 14% $MeOH/CH_2Cl_2$, were pooled and reduced *in vacuo* to give 600 mg (29% yield) of the desired trimer (rf 0.28 in 10% $MeOH/CH_2Cl_2$).

MALDI m/z 1492, calc'd. MNa^+ 1489.

^1H NMR (400 mHz, $CDCl_3$): δ 8.6 (d, 1H); 8.2 (d, 1H); 8.03 (t, 1H); 8.0 (d, 2H); 7.8 (t, 2H); 7.6-7.1 (m, 17H); 6.8 (m, 4H); 6.5 (t, 1H); 6.1 (s, 1H); 6.0 (m, 1H); 5.3 (broad s, 1H); 5.2 (s, 1H); 5.0 (broad s, 1H); 4.5-4.2 (m, 15H); 3.8 (s, 6H); 3.4 (m, 2H); 3.2 (m, 1H); 2.8-2.2 (m, 12H); 1.8 (m, 3H).

Example 4: **Preparation of N^6-Benzoyl-5'-0-(4,4'-dimethoxytrityl)de xyadenosine-3'-yl Cyanoethylphosphate-5'-yl Thymidine-3'-yl N^4-Benzoyldeoxycytidine-5'-yl Cyanoethylphosphate-3-0-(2-cyanoethyl)-N,N-diisopropylphosphoramidite**



where B^1 is C^{bz}, B^2 is T, and B^3 is A^{bz}.

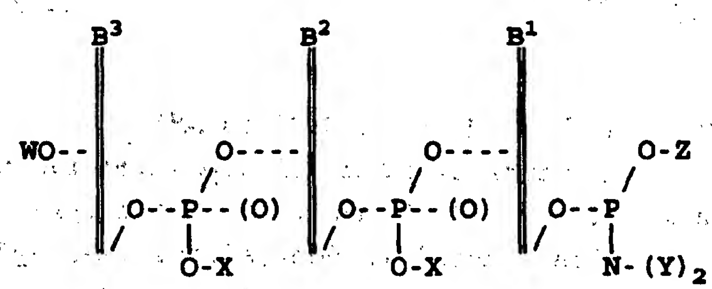
A mixture of 50 mL of CH_3CN and 100 ml THF were used to dissolve 400 mg (0.27 mmol) of N^6-benzoyl-5'-0-(4,4'-dimethoxytrityl)deoxyadenosine-3'-yl cyanoethylphosphate-5'-yl thymidine-3'-yl N^4-benzoyldeoxycytidine-5'-yl cyanoethylphosphate (from Example 3). The solution was stripped to a solid *in vacuo*. After argon was blown into the flask, the solid was re-dissolved in 60 mL THF. About 1 mL of diiso-propylethylamine (DIPEA) was added, along with 300 μL 2cyanoethyl-N,N-diisopropylchlorophosphoramidite (318 mg, 1.3 mm). The

solution was allowed to stand for 20 minutes, and 5 mL of 5% aqueous $NaHCO_3$ wer add d. Th solvent was removed *in vacuo*, and 100 mL of ethyl acetate was added. The solution was washed with 60 mL of a 5% aqueous solution of $NaHCO_3$, dried over Na_2SO_4, and reduced to a solid *in vacuo*. A silica column (2 X 7 cm) was prepared using 2% methanol and 1% pyridine in CH_2Cl_2. The crud product was applied to the column using 10 mL of this solvent. Elution proceeded with a series of $MeOH/CH_2Cl_2$ mixtures, specifically, 100 mL of 2% $MeOH/CH_2Cl_2$, 100 mL of 3%, 100 mL 4%, 100 mL 5% and 100 mL of 6% $MeOH/CH_2Cl_2$. The product-bearing fractions, which eluted at 6% $MeOH/CH_2Cl_2$, were pooled and reduced *in vacuo* to give 140 mg (31% yield) of the desired codon amidite (rf 0.45 at 10% $MeOH/CH_2Cl_2$).

^{31}P NMR (162 mHz, $CDCl_3$): δ 8 147.2, 147.16, (P^{III}), -4.560, -5.563 (P^{V}). Area ratio P^{III} / P^{V} = 20.243 / 39.782.
Anal. Calc'd for $C_{82}H_{97}N_{14}O_{20}P_3 \cdot CH_2Cl_2$: C, 56.24; H, 5.58; N, 11.19. Found: C, 55.91; H, 5.23; N, 11.32.

Example 5: Preparation of Other Codon Phosphoramidite Compounds

The following additional trinucleotide codon phosphoramidites made by the general methods described above in Examples 1-4.



wherein W is DMT, X and Z are both $-CH_2CH_2CN$, and Y is diisopropyl amine;

‖ is the residue f a ribose or deoxyribose; and B^1, B^2 and B^3 for Compounds 1-4 are as shown below:

Compound 1: $B^3 = A^{bz}$; $B^2 = T$; and $B^1 = A^{bz}$

Compound 2: $B^3 = T$; $B^2 = T$; and $B^1 = C^{bz}$

Compound 3: B^3 = G^{ibu}; B^2 = T; and B^1 = A^{bz}

Compound 4: B^3 = C^{bz}; B^2 = G^{ibu}; and B^1 = A^{bz}

Example 6: DNA Synthesis and Analysis

The codon amidite triplets dATA, dCTT, dATC, dATG and dAGC were made by solution phase methods and converted into codon 3'-phosphoramidites, with protecting groups that are fully compatible with automated phosphoramidite DNA synthesis chemistry. The 3'-hydroxyls were converted into N,N-diisopropyl-β-cyanoethylphosphoramidites using the general method described above in Examples 1-4. The resulting codon amidites were single spot by thin layer chromatography (TLC), and two of the compounds appeared to be more than 90% pure by ^{31}P NMR.

These individual codon amidites were subjected to several functional tests. First, the individual codons were used to make a 16-mer dATACTTATCATGAGCC by sequential coupling onto a nucleoside-bearing, dC-functionalized solid support, using semi-manual methods. A Biosearch 8750 four-column, automated DNA synthesizer, running a synthesis program without the coupling step, was used for detritylation, oxidation and capping, as well as washing between steps.

Coupling was done manually in the following manner. Under argon, the codon amidite was dissolved in acetonitrile to make a 100 mg/mL solution, and 100 μL of this solution was taken up in a 1-cc tuberculin syringe (luer slip-tip). Tetrazole was dissolved in CH_3CN to make a 0.6 M solution, of which 100 μL was taken up in a second syringe.

The DNA synthesizer instrument was paused before the time that coupling would normally occur, and the liquid was removed from the column by an argon drying step. The column was removed from the instrument, and one syringe was inserted into each end of the column. The syringe containing the amidite was discharged into the column. The excess volume was allowed to enter the syringe containing the tetrazole solution at the opposite end. Next, the tetrazole-containing syringe was discharged, and the first syringe was allowed to fill with

excess mixed fluid. The process was repeated three times, and the column was allowed to stand for 1 minute.

After the syringes were removed, the column was plac d back on the instrument. Oxidation, capping and detritylation steps were performed as appropriate to complete the desired DNA structure. At the end of the synthesis, the DNA fragment was cleaved from the solid support and, at the same time, the exocyclic amine and internucleotide phosphate protecting groups were removed by treating the DNA product with concentrated NH_4OH for 72 hours at room temperature. Oligopac reverse phase cartridges (commercially available from Millipore Corp.) and the associated protocols for these cartridges were used for purification. On a 0.2 μM synthesis scale, 2 O.D. units (260 nm) of DNA were obtained after the cartridge purification procedure.

The same sequence was then made with monomers and, when tested by co-elution on SAX HPLC, the DNA fragment obtained appeared to be identical to the sequence made with monomers. See Figures 1A, 1B and 1C. The details of the SAX HPLC analysis are as follows: A 5 X 250 mm column of Whatman Partisil-10 was used. Buffer A, 0.002 M phosphate in 20% CH_3CN at pH 6.8, and Buffer B, 0.2 M phosphate in 20% CH_3CN at pH 6.8, were both prepared. The gradient used was 0-75% Buffer B over a 30-minute period, at a flow rate of 1.5 ml/min. The product was detected at 260 nm.

Snake venom phosphodiesterase/calf alkaline phosphatase digests of both 16-mers also looked identical, further establishing the structural integrity of the DNA fragments made by the method of the invention.

Next, to evaluate the relative coupling efficiency of the codon amidites, a roughly equimolar mixture of five different codons was coupled to a dC- functionalized solid support. Product tetramers made by coupling the codon amidites were quantified by analytical HPLC. The coupling efficiency of the codon amidite mixture averaged 71%, as compared to the 95-99% c upling efficiency observed for monomers.

The results of co-injection with tetramer standards prepar d with monomers showed substantial incorporation of four

out of five trimer c d n amidites being tested and confirmed that the identity of each of the tetramers produced was consistent with the intended reaction.

For calculation of the relative amounts of tetram rs in the HPLC trace, molar extinction coefficients for the nucleoside monomers were measured in 50% aqueous methanol at 260 nm: dA (13000); dG (11100); T (7550); dC (4700). These valu s were used to correct the areas used to represent the mole % of each species present: CTTC (30.2%); ATCC (46.5%); ATGC (10.0%); and AGCC (13.2%).

Example 7: Recombinant DNA Methods

The codon amidites were then used to produce a "scrambled region", in which 15 internal base pairs were randomized by coupling a mixture of all five codons (dATA, dCTT, dATC, dATG and dAGC) at five sequential sites. These scrambled regions were flanked by homologous regions made with amidite monomers. The procedure yielded a pool of 54-mer fragments with the sequence shown below in Table 1 as "Codon Amidite 1."

TABLE 1: DNA Sequences Used

Name	Sequence
GST-P1 SfiI	5'-CATGCCATGACTCGCGGCCCAGCCGCC-CATGGCATGCCTCCATACACAGTTGTTTA-3'
Codon Amidite 1	5'-CCAGCATTCTGCGGCCGC(XXX)$_5$GGGGA-GGTTCACGTACTCAGG-3'

The DNA was purified on a reverse phase cartridge. For the reverse phase analysis, a YMC Corp. C-18 column (5 X 250 mm) having a particle size of 5μ was used. Buffer A was 5% MeOH in 0.001 M TEAA, pH 7.5. Buffer B was 90% MeOH in 0.001 M TEAA, pH 7.5. The gradient was 0-100% Buffer B over 30 minutes, with product being detected at 260 nm. With 0.2 μM of support-immobilized initial nucleoside, 5 O.D. units of purified 54-mer were obtained. The oligonucleotide was further purified by denaturing urea/acrylamide gel electrophoresis.

This resulting 54-m r pool was used as a primer in the PCR amplification of cDNA encoding a human glutathione

S-transferase. Th human glutathi ne S-transferase P1-1 cDNA in the expression vector pKXHP1 was used as a PCR template, in accordance with the procedure of Widersten, Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science 383, Uppsala ISBN 91-554-9240-8 (1992). As a preliminary step, to eliminate an internal *SfiI* site at position 573-585 in the human P1-1 cDNA, overlap PCR mutagenesis was performed. A G-to-A substitution at position 582 removed the *SfiI* site, while leaving the amino acid sequence unchanged.

For randomization of amino acids corresponding to position 204-210 (INGNGKQ), the following amplification was performed with the codon amidite primer pool. The PCR reaction contained 25 pmol each of the primers GST-P1 *SfiI* and Codon Amidite 1 (shown above in Table 1), Perkin-Elmer *Taq* polymerase buffer with $MgCl_2$ added to 2 mM, 10 ng of template pKXHP1, all four dNTP's at 250 μM each, and 2.5 units of *Taq* polymerase, in a final volume of 50 μl.

Using an Omnigene thermal cycler, the reaction mixes were put through 25 cycles of denaturation (94°C, 1 minute); annealing (65°C, 1 minute); and extension (72°C, 1 minute); followed by a final cycle of extension (72°C, 10 minutes). The reaction product was gel purified, digested with *SfiI* and *NotI*, and gel purified once again.

The digested cDNA pool was subcloned into a phagemid vector by ligating to 20 ng *SfiI/NotI*-restricted pHEN-1 phagemid vector in a standard ligation reaction. Ligated phagemid DNA was then electrotransformed into TG-1 by the procedure of Hoogenboom *et al.*, Nuc. Acids Res., 19, 4133-37 (1991). After insertion of the mutagenized cDNA pool into the phagemid vector, sixteen transformants were randomly selected from this mini-library and were sequenced to evaluate the incorporation of the scrambled codon region. The recombinant clones were sequenced through the randomized region using the primer CTATGCGGCCCCATTCA in the dideoxy chain-termination method of Sanger *et al.*, Proc. Natl. Acad. Sci. USA, 74, 5463-67 (1977).

These clones showed random incorporation of the proper codon s quenc s at the correct location. Table 2 below shows all of the codon sequences read in the 16 clones.

TABLE 2

Clone	Sequence
1	ATC-ATG-ATG-ATC-CTT
2	ATG-ATC-ATC-ATG-CTT
3	AGC-AGC-ATC-CTT-ATC
4	ATC-ATG-ATC-ATC-ATC
5	AGC-CTT-ATC-ATC
6	ATC-ATC-ATC-CTT
7	ATC-CTT-ATC-ATC
8	ATG-CTT-ATC-CTT
9	AGC-ATC-ATC-CTT
10	CTT-CTT-ATC-AGC
11	AGC-ATC-ATC-ATG-CTT
12	ATG-ATC-CTT-ATC
13	ATC-CTT-ATC-CTT
14	AGC-ATC-CTT-CTT-ATC
15	ATC-ATA-ATC-ATC
16	ATC-ATC-AGC-ATG

Table 3 below lists the frequency and relative amounts of each codon transcript expressed.

Codon Amidite	Transcript Seen	Frequency	Rel. %
ATA	TAT	1	1.4
CTT	AAG	17	24.6
ATC	GAT	34	43.9

ATG	CAT	9	13.0
AGC	GCT	8	11.6

Thus, the transcribed base sequences in the scrambled region matched those expected from the codon amidites, with reasonabl correlation with the values obtained by the tetramer HPLC analysis above.

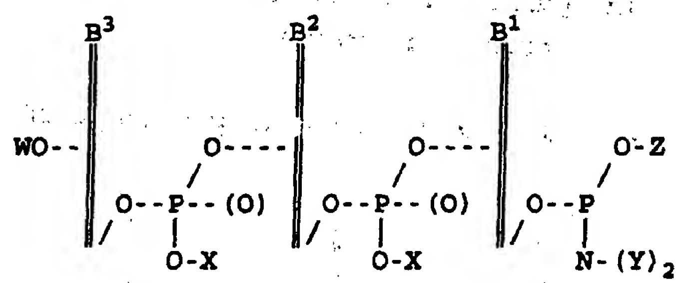
Example 8: Altering Proportions of Amidites to Improve Representation

In this experiment, the same procedures used in Example 7 were repeated, except that the concentration of the more reactive codon amidites was lowered in an attempt to make expression of each codon more equal, based on the frequencies seen above in Example 7. Four out of 19 randomly selected clones had full length library regions. The lower expression quality was believed to be a function of lower overall codon amidite concentration. However, the results did show the desired increase in dATG and decrease in dATC, establishing that changing the relative concentration of the codon amidites in the coupling mixture has a serviceable effect on expression. Taking all of the data together, the expression ratios of codons in th clones were closely mirrored by the tetramer model coupling.

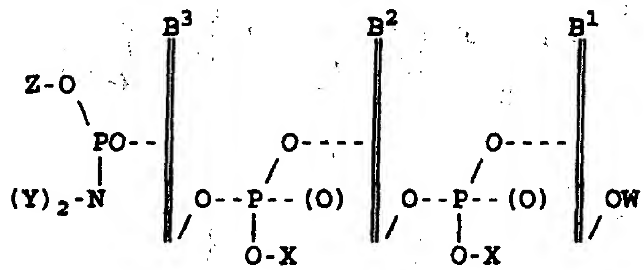
The inventors have therefore demonstrated the ability to construct desirably diversified DNA libraries using pre-formed triplet codon amidites and conventional phosphoramidite DNA synthesis.

W claim:

1. A compound of the formula:



or



wherein W, Y and Z are each independently hydrogen or a protecting group;

X is $-CH_2CH_2CN$;

B^1, B^2 and B^3 are each independently a base selected from the group consisting of protected adenine, protected guanine, protected cytosine, protected or unprotected thymine and protected or unprotected uracil; and

‖ is the residue of a ribose or deoxyribose.

2. The compound of claim 1 wherein W is DMT (dimethoxytrityl); and/or

wherein Y is an isopropyl group; and/or

wherein Z is $-CH_2CH_2CN$; and/or

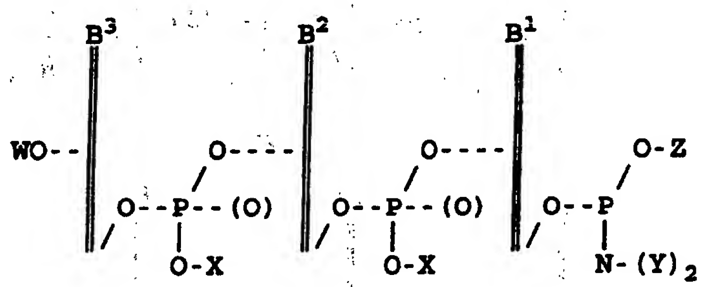
wherein B^1 is selected from the group consisting of protected adenine, protected guanine, protected cytosine, and protected or unprotected thymine.

3. The compound of claim 2 wherein any adenine base is protected with a benzoyl protecting group; and/or

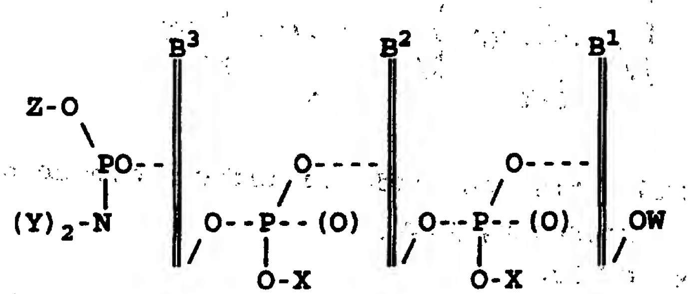
any cytosine base is protected with a benzoyl protecting group; and/or

any guanine base is protected with an isobutyryl protecting group.

4. A process for making a compound of the formula:



or



wherein W, X, Y and Z are each independently hydrogen or a protecting group;

B^1, B^2 and B^3 are independently a base selected from the group consisting of protected adenine, protected guanine, protected cytosine, protect d or unprotected thymine and protected or unprotected uracil; and

‖ is the residue of a ribose or deoxyribose,

comprising the steps of:

a. treating a nucleoside comprising a base, a ribose or deoxyribose residue, and a first protecting group at the 5' position or the 3' position, with a trialkylsilyl halide to produce the corresponding 3'- and 5'-substituted nucleoside;

b. removing the first protecting group at the 5' or 3' position to produce a 5' or 3' deprotected, 3' or 5'-substituted nucleoside;

c. coupling the 5' or 3' deprotected, 3' or 5' substituted nucleoside with a first nucleoside 3' or 5' phosphoramidite and then oxidizing to form a phosphate triester;

d. deprotecting at the 5'- and 3'-termini to give the corresponding 3',5'-dihydroxy dinucleoside;

e. coupling the dihydroxy dinucleoside with a second nucleoside 3' or 5' phosphoramidite and oxidizing to produce the two corresponding 3' or 5' hydroxy trinucleotides;

f. separating away unwanted products; and

g. converting the 3'- or 5'-hydroxy trinucleotide to a 3'- or 5' phosphoramidite.

5. The process of claim 4 wherein said removing step "b." is conducted under acid conditions; and/or

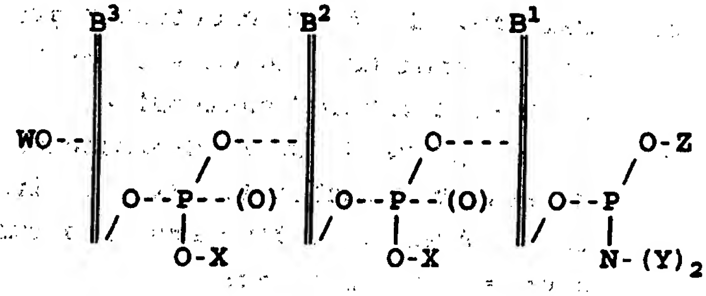
wherein, in said removing step "b.", acetonitrile is used as a solvent; and/or

wherein, in said removing step "d.", an alcohol or acetonitrile is used as a solvent; and/or

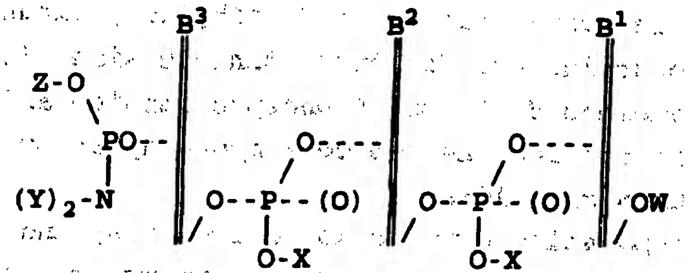
wherein, in step "d.", KF is added to the reaction mixture.

6. A panel of from two to twenty 3'- or 5'-phosphoramidite trinucleotide codons or their complements, wherein each codon encodes a different amino acid.

7. The panel of claim 6 wherein each codon has the formula:



or



wherein W, X, Y and Z are each independently hydrogen or a protecting group;

B^1, B^2 and B^3 are independently selected from the group consisting of protected adenine, protected guanine, protected cytosine, protected or unprotected thymine and protected or unprotected uracil; and

‖ is the residue of a ribose or deoxyribose.

8. The compound of claim 7 wherein W is DMT (dimethoxytrityl); and/or

wherein X is $-CH_2CH_2CN$; and/or

wherein Y is an isopropyl group; and/or

wherein Z is $-CH_2CH_2CN$.

9. A process for synthesizing an oligonucleotide encoding a sequence of "n" pre-determined amino acids, or its complement, comprising the steps of:

(a) coupling a trinucleotide codon, or its complement, onto a solid support;

(b) sequentially condensing through a phosphoramidite linkage with each immediately pr ceding codon, or complement,

n-1 trinucle tide codons or their complements, each codon or its complem nt corresponding to the next pre-determined amino acid in the sequence; and

(c) cleaving the oligonucleotide from the solid support.

10. A process of synthesizing an oligonucleotide encoding a peptide having at least one pre-determined amino acid position and at least one random amino acid position, comprising the steps of:

(a) coupling a first trinucleotide codon, or the complement to said codon, onto a nucleoside- or nucleotide-bearing solid support;

(b) for each pre-determined amino acid position, sequentially coupling, through a phosphoramidite linkage, to an immediately preceding codon, or complement, a trinucleotide codon, or its complement, corresponding to the pre-determined amino acid;

(c) for each random amino acid position, coupling through a phosphoramidite linkage, to an immediately preceding codon or complement, a mixture of from two to twenty trinucleotide codons, at least two of the trinucleotides representing a codon to a different amino acid; and

(d) cleaving said nucleotide from the solid support;

wherein steps (b) and (b) are combined in such a way that each pre-determined codon sequentially corresponds to each pre-determined amino acid position in said oligonucleotide and

wherein the identity and ratio of codons used in each said mixture are representative of the degree of diversity desired in the corresponding random amino acid position of said oligonucleotide.

11. A pr c ss of synthesizing an oligonucleotide having at least one region of random amino acid positions, of the invention comprises the steps of:

(a) coupling at least one pre-existing trinucleotide codon, or complement, onto a nucleoside- or nucleotidebearing solid support;

(b) for each random amino acid position, sequentially coupling, through a phosphoramidite linkage, to the immediately preceding codon, or complement, a mixture of from two to twenty pre-existing trinucleotide codons, each codon corresponding to a different amino acid; and

(c) cleaving the oligonucleotide from the solid support;

wherein the identity and ratio of codons used in each said mixture are representative of the degree of diversity desired in the corresponding random amino acid position of said oligonucleotide.

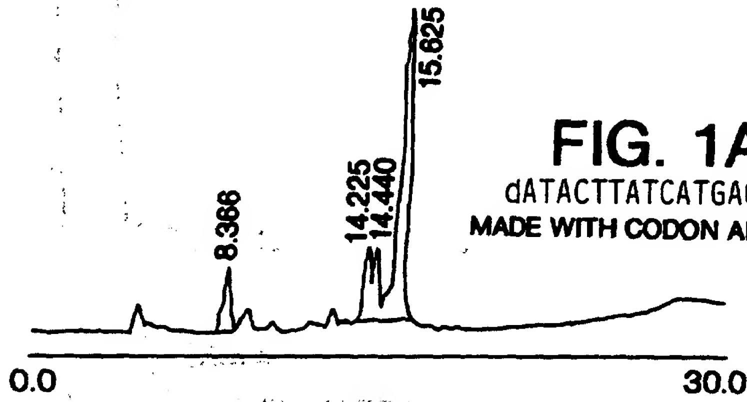


FIG. 1A
dATACTTATCATGAGCC
MADE WITH CODON AMIDITES

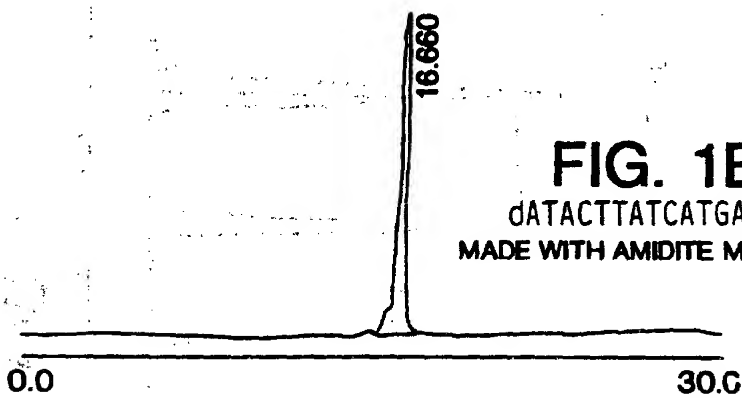


FIG. 1B
dATACTTATCATGAGCC
MADE WITH AMIDITE MONOMERS

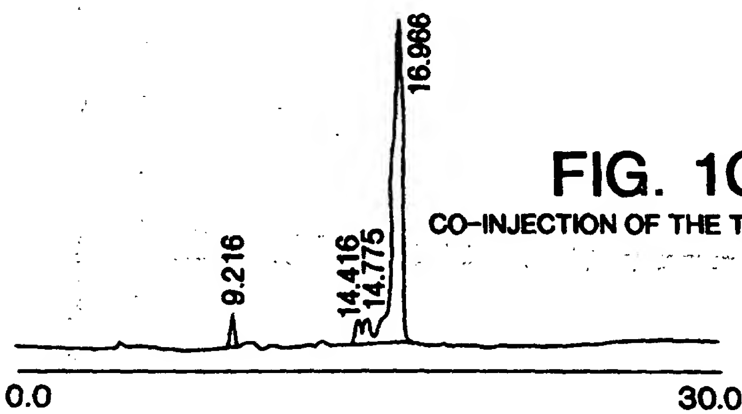


FIG. 1C
CO-INJECTION OF THE TWO FRAGMENTS

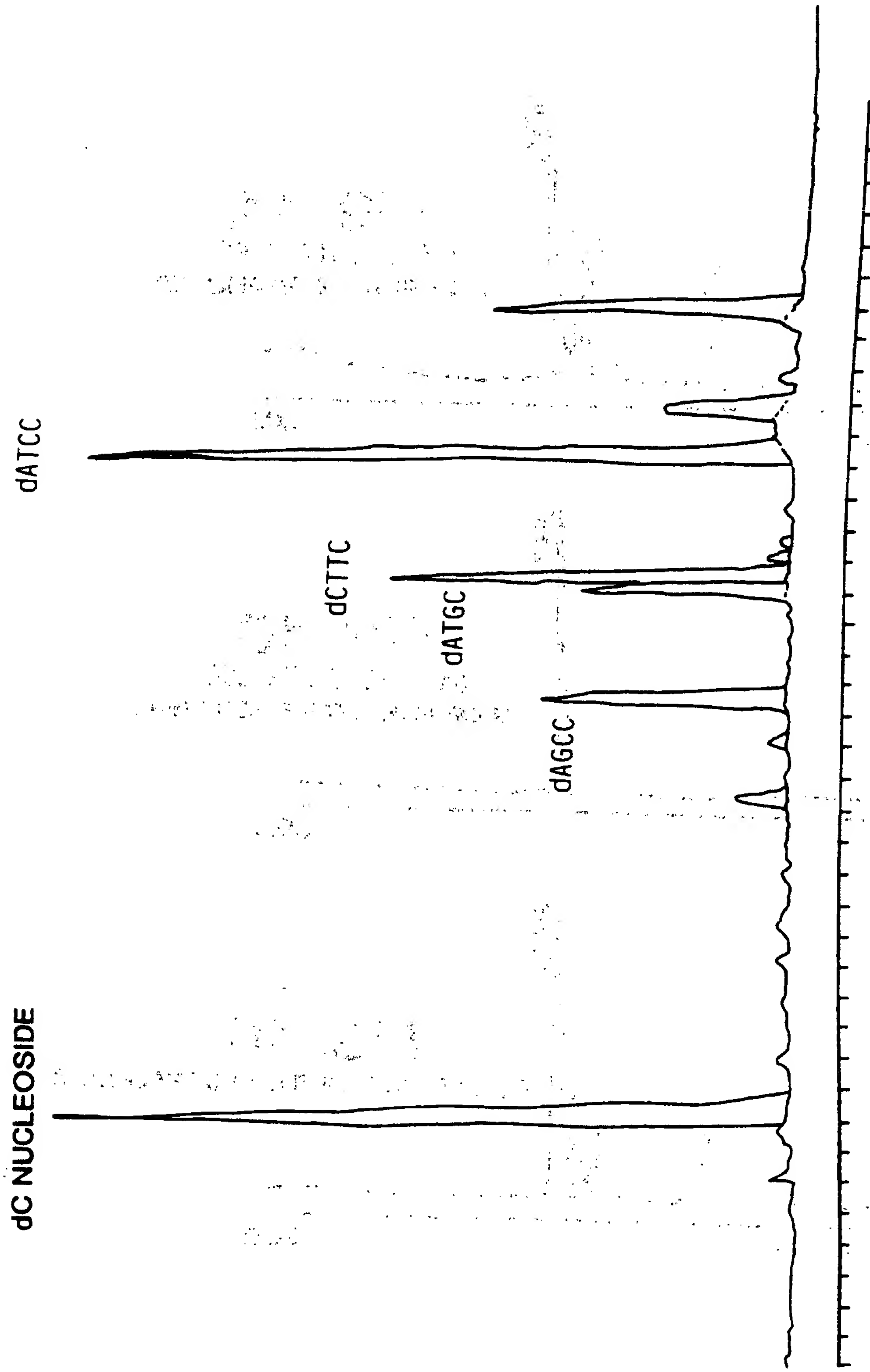


FIG. 2

[illegible]

[illegible]

[illegible]

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